Award Number: W81XWH-11-1-0671

TITLE:

The Risk and Clinical/Molecular Characteristics of Breast Cancer in Women with Neurofibromatosis Type 1

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REPORT DATE: October 2014

TYPE OF REPORT: ANNUAL REPORT

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2014	Annual report	30 SEP 2013 - 29 SEP 2014

4. TITLE AND SUBTITLE

"The Risk and Clinical/Molecular Characteristics of Breast Cancer in Women with Neurofibromatosis Type 1"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0671

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Henry Ford Health System
Detroit, Michigan 48202

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The purpose of the project is to characterize the breast cancer in women affected with Neurofibromatosis type 1 (NF1) in a multi-institutional setting. Aim 1 assessed the incidence of breast cancer in this cohort and the clinical features of NF1 associated with breast cancer and other cancers. Aim 2 investigated the NF1 gene germline mutations in women with breast cancer. A total of 423 cases of NF1 women have been reviewed. History of breast cancer was found in 20. Family history of cancer is associated with a personal history of breast cancer ($p=6.05x10^{-5}$). No other clinical feature or family history is found to be significantly associated with the occurrence of breast cancer. Malignant peripheral nerve sheath tumor (MPNST) is associated with plexiform neurofibromas; p=0.049. Learning disability is associated with CNS tumor and/or optic glioma (OPG); p=0.012. European Americans (EA) are more likely to develop CNS tumor and/or OPG than African Americans (AA); p=0.002. The rate of OPG alone is also higher in EA than AA; p=0.013. Breast cancer is not significantly more prevalent in EA or AA. However, with breast cancer excluded, more EA individuals developed other cancers than AA, p=0.032. To date, germline NF1 mutations has been investigated in 14 women with breast cancer history. Four (28%) are mutations affecting in-frame splicing, possibly more than average NF1 cases. Aim 3 has not been completed yet. Aim 4. NF1 inactivation results in human mammary epithelial cells (HMEC) senescence; p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC; p53 inactivation provides an initial growth advantage to HMEC with a consequent large number of cell death; Overexpression of K-Ras V12 does not transform p53 inactivated HMEC.

15. SUBJECT TERMS
NF1; breast cancer; signaling pathway; germline mutation; somatic mutation; FFPE; IHC; ion semiconductor sequencing; whole exome sequencing, NF1 knockdown cells; senescence, Ras

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	34	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Occurrence of breast cancer is increased in women affected with Neurofibromatosis type 1 (NF1). This study is aimed at identifying an accurate incidence of breast cancer in this group of women in a multi-center collaborative environment. There are 4 specific aims. **Aim 1** is to confirm the increased breast cancer risk in women with NF1. All the participating centers, Henry Ford Health System (HFHS), University of Alabama at Birmingham (UAB), Children's National Medical Center in D.C. (CNMC), and Johns Hopkins University (JHU), have reviewed the medical records of women affected with NF1. Clinical data were analyzed to identify clinical features associated with the occurrence of breast cancer. Clinical features were also analyzed for their association with other type of cancers in this study. At the same time, women with a history of breast cancer were recruited to donate blood and their archived tumor specimen. **Aim 2** is to analyze the germline *NF1* gene and whole exome sequencing data in the subjects with history of breast cancer. The NF1 mutations identified were analyzed for genotype-breast cancer correlation. Additional germline gene changes may reveal germline breast cancer predisposition in addition to the NF1 gene. **Aim 3** is to determine if NF1 associated breast cancers have unique characteristic signaling pathways or molecular tumorigenesis. Immunohistochemistry study of the signaling pathway will be performed on archived tumor blocks. NF1 gene mutation or copy number variation will be analyzed on these tumor specimens. Next generation sequencing of a 31 gene panel known to be associated with breast cancer will be performed as well. The NF1 gene is included in the panel. **Aim 4** is to study the phenotype of NF1 knockdown in primary mammary epithelial cells, specifically focused on the senescence effect due to Ras activiation. This study attempts to provide information in determining when and how to screen for breast cancer in this group of women. It will also shed light on the molecular mechanisms of breast cancer in NF1 deficient human subjects.

KEY WORDS

NF1; breast cancer; signaling pathway; germline mutation; somatic mutation; FFPE; IHC; ion semiconductor sequencing; whole exome sequencing, NF1 knockdown cells; senescence, Ras

OVERALL PROJECT SUMMARY

A multicenter case review was conducted for women affected with neurofibromatosis type 1 (NF1). The focus of this review is to explore correlation between occurrence of malignant neoplasms and clinical features as well as family history.

The case review has been conducted by the Henry Ford Health System in Detroit, the neurofibromatosis clinic in the University of Alabama at Birmingham and the neurofibromatosis clinic in the Johns Hopkins University. The neurofibromatosis clinic in the Children's National Medical Center, Washington D. C. contacted the affected mothers of the children with NF1 and collected relevant medical information after informed consent.

All centers recruited NF1 women with a history of breast cancer to donate blood and tumor specimens for analysis.

In Wayne State University, Dr. Tainsky's laboratory, the senescence effects were studied for its response to hyper-activated Ras via NF1 knockdown on human mammary epithelial cells (HMEC).

Aim 1. To identify clinical features associated with the risk of breast and other cancers.

Methods:

The medical records are reviewed for all the women whose age are 20 years or older at the time of study. The individual has to meet the clinical diagnostic criteria for NF1 or the diagnosis has been confirmed by a deleterious mutation in NF1 gene.

The cases reviewed include all the women presented at outpatient visit encounters during the following periods of time:

1. Henry Ford Health System: from 1994 to 2013

2. University of Alabama at Birmingham NF clinic: from 2011 to 2013

3. Johns Hopkins University NF clinic: from 2003 to 2013

4. Children's National Medical Center NF clinic: The adult woman NF1 subjects were recruited when they brought their affected children to the NF clinic for evaluation. The time period is from 2011 to 2013.

The information collected includes the date of birth, ethnicity, and biological relationship within the cohort. Medical information collected include numbers of café au lait macules on the skin, skin fold freckling, Lische nodules on the irises, number of dermal neurofibromas, number of plexiform neurofibromas, history of optic gliomas, malignant peripheral nerve sheath tumor, bony dysplasia, macrocephaly, short stature and learning disability. Additional information regarding neoplasm collected include occurrence of any malignant solid tumors, malignant hematological disorder, tumor of central nervous system (CNS). For breast cancer, pathological type, stage and age at diagnosis are collected. Breast cancer screening and biopsy information are also collected. Family history information collected includes NF1, malignant neoplasm, CNS tumor, and number of relatives with breast cancer. Genetic test results such as NF1 gene mutation and/or BRCA1 and BRCA2 mutation are documented when available.

The occurrence of central nervous system (CNS) tumors and types of malignant neoplasms are analyzed for their possible correlation with clinical features frequently seen in individuals affected with NF1. The malignant neoplasms are grouped based on their epithelial, endothelial, and mesodermal origin. CNS tumor category includes all categories, from low grade glioma to high grade glioblastoma. Optic glioma is included. Because of the unknown significance, the feature of thickened optic nerve or chiasm alone is excluded from the category of optic glioma (OPG).

Statistical analysis (chi-square tests or Fisher's exact tests) was performed to detect any association between each clinical feature of NF1 and incidence of cancer.

Results:

A total of 423 cases of women affected with NF1 are reviewed. The study sample included 250 European Americans (Caucasians), 118 African Americans, and 41 individuals with other ethnicities. Ethnicity information is not available for 14 women. Among them, 36 women are related to at least another woman in this cohort. They belong to 16 families. The case ratio between European Americans and African American are rather consistent through age groups: 71:35, 59:29, 48:21 and 72:33 in age groups of 20-29, 30-39, 40-49 and 50 years or older, respectively.

1. Breast Cancer:

A total 20 women have had history of breast cancer. Among them, 11 are European American, 8 are African American. Ethnicity of the remaining one is not available. Half of the cases (n=10) were diagnosed between 40 to 49 years of age. A quarter of the cases (n=5) were diagnosed between 30 to 39 years of age. (Table 1). Two cases were affected with additional primary breast cancer after the first diagnosis. All of these breast cancers are ductal carcinoma, except one ER positive invasive lobular carcinoma.

Table 1. Breast Cancer Pathology

	Invasive (n)	**In Situ** (n)	**N/A** (n)
Total	**8**	**6**	**8**
Ductal	6	6	9
ER+	4	0	15
ER-	2	1	
Her2+	1	1	17
Her2-	3		
Stage			
1	**2**		
2	**1**		
3	**5**		
4	**0**		

2. **Occurrence of Breast Cancer and Family History**

The rate of personal history of breast cancer is strongly associated with family history of all cancers (14.3% 12/84 v.s. 2.37%, 8/338; p=0.00006). (Table 2)

The personal breast cancer may be associated with family history of breast cancer within 3rd degree female relatives. In women with family history of breast cancer, 16.67% (2/12) had breast cancer. The rate is higher than those without family history, 3.3% (6/182). However, this association has not reached statistical significance (p=0.080).

In addition, the personal breast cancer is not significantly associated with family history of NF1. None of the women with breast cancer have relatives with both NF1 and breast cancer.

Table 2. Breast Cancer and Family History

	No Breast Cancer	Breast Cancer	%	P value
No Family History of Any Cancer	330	8	2.37	6.05×10^{-5}
Family History of Any Cancer	72	12	14.30	
No Family History of Breast Cancer	176	6	3.3	0.080
Family History of Breast Cancer	10	2	16.67	
Information Not Available	217	12		
No Family History of NF1	209	6		0.126
Family History of NF1	152	10		

3. Occurrence of Breast Cancer and Clinical Features of NF1

After excluding the cases with unavailable features, the association between NF1 features and breast cancer or all cancers are analyzed. The rate of breast cancer or all cancers has no statistically significant association with any clinical features, such as the number of café au lait macules, the number of dermal neruofibromas, Lische nodules, plexiform neurofibroma, skeletal dysplasia, short stature, vasculopathy, macrocephaly, or learning disability. (Table 3)

Table 3. Occurrence of Breast Cancer or All Cancers and Clinical Features of NF1

			No Cancer		Cancer			No Cancer		Cancer		
Character-istic Features	**Cancer**	**Feature Catego-ries**	**N**	**%**	**N**	**%**	**P**	**N**	**%**	**N**	**%**	**P**
Café au lait	All Cancer	0-5	33	10.34	6	5.71	0.32	33	11.07	6	6.25	0.237
		>=6	266	83.07	89	85.71		266	88.93	89	93.75	
		Missing	21	6.58	9	8.57						
	Breast Ca	0-5	38	9.45	1	4.55	0.111	38	10.11	1	5.56	1
		>=6	340	84.08	15	77.27		340	89.89	15	94.44	
		Missing	26	6.47	3	18.18						
Lisch Nodule	All Cancer	0	105	32.92	34	32.38	0.462	105	51.47	34	46.58	0.498
		>=1	100	31.03	38	37.14		100	48.53	38	53.42	0
		Missing	115	36.05	32	30.48						
	Breast Ca	0	137	34.08	2	9.09	0.001	137	50.55	2	33.33	0.447
		>=1	136	33.33	2	18.18		136	49.45	2	66.67	0
		Missing	131	32.59	15	72.73						
Dermal Neuro-fibroma	All Cancer	0-1	45	14.11	12	11.43	0.348	45	14.61	12	11.54	0.513
		>=2	264	82.45	91	87.62		264	85.39	91	88.46	
		Missing	11	3.45	1	0.95						
	Breast Ca	0-1	55	13.68	2	9.09	0.871	55	14.1	2	9.09	0.753
		>=2	337	83.33	18	90.91		337	85.9	18	90.91	
		Missing	12	2.99	0	0						
Plexiform Neuro-fibroma	All Cancer	0	172	53.92	58	55.24	0.851	172	61.87	58	61.7	1
		>=1	107	33.23	35	34.29		107	38.13	35	38.3	
		Missing	41	12.85	11	10.48						
	Breast Ca	0	218	54.23	12	54.55	0.055	218	61.24	12	75	0.306
		>=1	140	34.33	2	18.18		140	38.76	2	25	
		Missing	46	11.44	6	27.27						
Learning Disability	ALL Cancer	No	172	53.61	46	44.76	0.268	172	70.08	46	61.04	0.162
		Yes	73	22.88	30	28.57		73	29.92	30	38.96	
		Missing	75	23.51	28	26.67						
	Breast Ca	No	212	52.24	6	36.36	0.026	212	67.74	6	72.73	1
		Yes	100	24.88	3	13.64		100	32.26	3	27.27	
		Missing	92	22.89	11	50						

Table 3. Occurrence of Breast Cancer or All Cancers and Clinical Features of NF1…continued

			No Cancer		Cancer			No Cancer		Cancer		
Character-istic Features	Cancer	Feature Catego-ries	N	%	N	%	P	N	%	N	%	P
Vascu-lopathy	All Cancer	No	242	75.5	73	70.48	0.47	242	95.63	73	96.1	1
		Yes	11	3.45	3	2.86		11	4.37	3	3.9	0
		Missing	68	21	27	26.67						
	Breast Ca	No	307	75.87	8	45.45	0.002	307	95.61	8	100	1
		Yes	14	3.48	0	0		14	4.39	0	0	0
		Missing	84	20.65	11	54.55						
Scoliosis	All Cancer	No	202	63.01	66	63.81	1	202	73.9	66	74.44	1
		Yes	72	22.26	22	21.9		72	26.1	22	25.56	0
		Missing	47	14.73	15	14.29						
	Breast Ca	No	259	64.18	9	45.45	0.023	259	74.14	9	71.43	0.763
		Yes	91	22.39	3	18.18		91	25.86	3	28.57	0
		Missing	54	13.43	8	36.36						
Short Stature	All Cancer	No	129	40.75	43	41.9	0.538	129	76.47	43	72.13	0.494
		Yes	40	12.54	17	16.19		40	23.53	17	27.87	
		Missing	151	46.71	42	41.9						
	Breast Ca	No	164	40.8	10	45.45	0.539	164	74.55	10	90.91	0.301
		Yes	56	13.93	1	4.55		56	25.45	1	9.09	
		Missing	184	45.27	9	50						
Macro-cephaly or learning disability	All Cancer	No	147	46.08	45	42.86	0.849	147	55.47	45	52.33	0.62
		Yes	118	36.99	41	39.05		118	44.53	41	47.67	
		Missing	55	16.93	18	18.1						
	Breast Ca	No	184	45.77	8	36.36	0.003	184	54.28	8	66.67	0.558
		Yes	155	38.56	4	18.18		155	45.72	4	33.33	
		Missing	64	15.67	9	45.45						
Bony Dysplasia	All Cancer	No	273	85.58	87	82.86	0.259	273	94.46	87	89.69	0.157
		Yes	16	5.02	10	9.52		16	5.54	10	10.31	
		Missing	30	9.4	8	7.62						
	Breast Ca	No	345	85.32	15	77.27	0.4	345	93.46	15	89.47	0.371
		Yes	24	5.97	2	9.09		24	6.54	2	10.53	
		Missing	35	8.71	3	13.64						

4. Breast Cancer Screening:

The information for breast cancer screening is available for 31.44% (133 of 423) of the women. Among the women who received screening, 59.40% (79 of 133) are known to have had physical breast examination, 33.08% (44 of 133) had breast lumps, 23.30% (31 of 133) underwent biopsy. For breast imaging evaluations, about 48.12% (64 of 133) has had mammogram. Only 3.76% (5 out of 133) cases have undergone MRI evaluation for breast lumps. On the contrast, breast neurofibromas are recorded in 10% of the women in this cohort. Breast clinical examination with palpation or by mammogram cannot sufficiently distinguish a neurofibroma from a malignant breast mass. These data suggests that majority (70%) of the breast lumps were further evaluated by biopsy, but not by MRI.

5. MPNST and Plexiform Neurofibroma

This occurrence of MPNST is related to plexiform neurofibromas (PN). Among women with documented PN, 7.9% (11/139) have a history of MPNST, significantly higher than the women without documented PN, 3.14% (7/223); p=0.049. (Table 4)

Table 4. MPNST and Plexiform Neurofibroma

	No MPNST	**MPNST**	**%**	**p**
No PN	216	7	3.14	0.049
PN	128	11	7.91	

6. Learning disability, CNS tumor, optic glioma (OPG)

The rate of CNS tumor is higher in women with history of OPG comparing with the women without OPG, 12.2% (5/41) v.s. 4.48% (13/290); p=0.057. (Table 5)

Table 5. CNS tumor and optic glioma (OPG)

	No CNS tumor	CNS Tumor	%	p =
No OPG	277	13	4.48	0.057
OPG	36	5	12.2	

Learning disability is associated with history of CNS tumor and/or optic glioma. The women with learning disability has a higher rate of CNS tumor and/or OPG than the ones without learning disability, 23.3% (21/90) v.s. 11.23% (21/187); p= 0.012. (Table 6)

Table 6. Learning disability, CNS tumor and/or optic glioma (CNS+OPG)

	No tumor	Tumor	%	p =
No Learning Disability	166	21	11.23	0.012
Learning Disability	69	21	23.3	

When separating CNS tumor from optic glioma, the tumor continue to have higher rate in women with learning disability than the ones without. For CNS tumor without OPG, 4.85% (5/103) v.s. 0.92% (2/218); p=0.037. However the number contributed to the isolated CNS tumor analysis is very small. For OPG with or without CNS tumor, the analysis is not significant, 17.78%(16/90) v.s. 10.21%(19/186); p=0.085. (Table 7)

Table 7. Learning disability, CNS tumor or optic glioma (OPG)

	No CNS tumor	CNS tumor	%	p
No LD	216	2	0.92	0.037
LD	98	5	4.85	
	No OPG	OPG	%	p
No LD	167	19	10.21	0.085
LD	74	16	17.78	

Optic glioma mostly occurs in the early childhood. As therapy, chemotherapy or radiation is known to have adverse effects on the developing brain. One adverse effect is learning disability. The other adverse effect is that increased rate of CNS tumor later in life was reported in NF1 patients who underwent radiation therapy for OPG. The history of OPG treatment was not collected in this study. Therefore, the role of treatment contributing to the learning disability or CNS tumor later in life cannot be determined from this study.

7. **Dermal neurofibroma:**

We have not found a statistically significant association between the dermal neurofibroma load and the rate of various types of malignant neoplasms. (Table 3)

8. Ethnicity and Malignant Neoplasms

There are significant differences by ethnicity for "CNS+OPG" and "Other cancers". "CNS+OPG" refers to CNS tumors and/or OPG. "Other cancers" refers to all malignant tumors, hematological malignancies, CNS tumors and OPG, excluding breast cancer. The percentage positive for "CNS+OPG" and "Other cancers" is higher for European American than African Americans.

For the "CNS+OPG" definition, European Americans (EA) were significantly more likely to develop these tumors (21.24% 41/193) than African Americans (AA) (6.82% 6/88); p=0.002. The occurrence of OPG alone is also higher in European Americans (16.84% 32/190) than African Americans (5.68% 5/88); p=0.013. (Table 8)

Table 8. Ethnicity, CNS tumor or OPG

	No CNS+OPG	CNS+OPG	%	p
European American	152	41	21.24	0.002
African American	82	6	6.82	

	No OPG	OPG	%	p
European American	158	32	16.84	0.013
African American	83	5	5.68	

For the "Other cancers" definition, European Americans were significantly (p=0.032) more likely to develop these tumors (25.2% 63/250) than African Americans (15.25% 18/118). (Table 9)

Table 9. Ethnicity and other cancers

	No Other Cancers	Other Cancers	%	p
European American	187	63	25.2	0.032
African American	100	18	15.25	

Analysis could not demonstrate a statistically significant association between ethnicities and breast cancer. African Americans with 6.78% (8/118) v.s. European Americans with 4% (10/250); p= 0.301. (Table 10)

Table 10. Ethnicity and breast cancers

	No Breast Ca	Breast Ca	%	p
European American	240	10	4.00	0.301
African American	110	8	6.78	

Mesenchymal cancers (MPNST, GIST, and hematological malignancies) rate is not significantly higher in European Americans (8.8% 22/250) than African Americans (4.24% 5/118); p=0.137. (Table 11)

Table 11. Ethnicity and mesenchymal cancers

	No Mesen Ca	Mesen Ca	%	p
European American	228	22	8.8	0.137
African American	113	5	4.24	

The rate of MPNST and/or plexiform neurofibromas (PN) shows no significant difference among different ethnicities either.

However, the rate of epithelial and/or endothelial malignancies is trending higher in African Americans than European Americans without reaching statistical significance, 11.86% (14/118) v.s. 6%(15/250); p=0.062. Epithelial and endothelial cancer include cancers occurred in breast, lung, skin, thyroid, gastrointestinal tract, ovary, and endometrium. (Table 12)

Table 12. Ethnicity and epithelial and/or endothelial cancers

	No Epi/Endo Ca	Epi/Endo Ca	%	p
European American	235	15	6.00	0.062
African American	104	14	11.86	

9. Ethnicity and other clinical features:

Lische nodules are more common in European Americans (59%) relative to both African Americans (39%; p=0.009) and other ethnicities (32%; p=0.010). (Table 13)

	No LN	LN	%	p
European American	70	100	58.82	
African American	40	26	39.39	0.009
0ther Ethnicities	21	10	32.26	0.010

There is a significant difference (p=0.0027) in the percentage of individuals with 20 or more dermal neurofibromas (relative to 5 or less dermal neurofibromas) by ethnicity, with African Americans having a higher rate (75.81%, 47/62) of high dermal neurofibromas load than European Americans (53.03%, 70/132). (Table 14)

Table 14. Ethnicity and dermal neurofibroma load, all age group

	5 or Less	20 or more	%	p
European American	62	70	53.03	0.0027
African American	15	47	75.81	

This higher dermal tumor load in African Americans is manifested well within the age group of 20 to 39 years when it is compared with European Americans, however it has not reached the statistical significance; p=0.080. (Table 15)

Table 15. Ethnicity and dermal neurofibroma load, 20 – 39 years age group

	5 or Less	20 or more	%	p
European American	50	22	30.56	0.08
African American	11	20	64.52	

Aim 2. To analyze germline NF1 gene on the subjects with history of breast cancer. To perform germline whole exome sequencing (WES) on the subjects' blood specimens.

1. NF1 gene mutations carried by the NF1 women who developed breast cancer

There are 15 NF1 women recruited to participate in NF1 germline mutation analysis and submitted breast tumor specimens for further analysis if the tumors are retrieved. Nine of them were recruited from outside the 4 participating NF clinics. One case does not carry a NF1 mutation. In retrospective review, she does not really fit the minimum clinical diagnostic criteria, and therefore is excluded from further analysis. Two cases are from the same family. NF1 gene mutations/variants identified are shown on Table 16.

Mutations affecting in-frame splicing or exon skipping --	4 cases (28.6%)
Truncation mutation due to frame shifting or nonsense mutation --	9 cases (64.3%)
Mis-sense variant --	1 case (7.1%)

In-frame splicing or exon skipping types of mutation is relatively more than large cohort NF1 study (Sabbagh et al., 2013). This 2013 French cohort showed 6.5% in-frame splicing mutation, 65% truncating mutation and 7.5% missense mutation.

Table 16. NF1 gene mutation, breast cancer pathology and other information.

Study ID	Age	BrCa	Age at diag-nosis	Pathology				Mutation			BRCA gene testing
				ER	PR	Her 2	Ki67	Exon	Type	Description	
OP-1000	67	1	56	9	9	9		42(33)	Truncation	c.6364G>A, out-of-frame exon skipping	
OP-1001	60	1	53	1	1	2			Truncation	c.1541_1542delAG, truncating	negative
OP-1002	66	2	60	1	1	9		41	splicing	c.6792C>G (skipping exon 41)	negative
1070	62	1	49	2	2	1			Truncation	c.2398G>T, p.Glu800*, truncating	
1185	62	9		9	9	9		34 (26)	Truncation	c.4515-20_4515-18delAAG), out-of-frame splicing	
OP-1003	48	1	47	1	1	2		21	Truncation	(c.2621_2634dupAGGGTTCTATGATT), p.Ser879Argfs*4, truncating and splicing	negative
OP-1004	41	1	39	1	1	9		38-39 (29-30)	splicing	deletion of exons 38 and 39 (c.5206-?_5749+del?)	
OP-1005	52	1	41	1	2	3		16 (12a)	Mis-sense	c.1733T>G (p.Leu578Arg)	
OP-1006	50	1	49	1	2	1	3 (20%)	40 (39)	Truncation	c.5943+1G>T, out-of-frame splicing	negative
1299	54	1	44	1	1	2	3 (15%)	21(16)	Truncation	c.2728_2729delGG, truncating	
1300	58	9		9	9	9		37	Truncation	c.4910_4911delTT, truncating	
1095	53	1	52	2	2	1		39(30)	Truncation	c.5667dupT, truncating	
OP-1007	47	1	47	1 (24%)	1 (2%)	2	1 (59%)	11(9)	splicing	c.1260+1604A>G, in-frame splicing, deep intron	negative
OP-1008	52	1	42	1	1	2		11(9)	splicing	c.1260+1604A>G, in-frame splicing, deep intron	negative

2. **Whole exome sequencing of the germline genomic DNA has been completed.**

   Data analysis is ongoing at the present time.

Aim 3. To determine if NF1 associated breast cancers have unique signaling pathway and molecular tumorigenesis characteristics.

Thirteen tumor specimens have undergone micro-dissection.

1. IHC assay for pMEK, ERK, pERK, AKT, mTOR, p53. PTEN, Her2 and Ki67 are ongoing at the present time.

2. Tumor specimen molecular analysis by LOH and methylation assay for NF1, p53, BRCA1, BRCA2, PTEN, and ATM genes

   To be completed

3. Full gene sequencing on formalin-fixed paraffin-embedded (FFPE) tissue: NF1, BRCA1, BRCA2, TP53, PTEN, and ATM genes, and additional breast cancer gene targets including; CDH1, RB1, MLL3, MAP3K1, CDKN1B, PIK3CA, AKT1, GATA3 TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPN22, PTPRD, 3F3B1, and CCND.

   To be completed.

Aim 4:
Phenotypic Analysis of NF1 knockdown in normal mammary epithelial cells

These cell line studies have been conducted in Dr. Michael Tainsky's laboratory in Wayne State University.

In order to study the association of Neurofibromatosis Type I to breast cancer development we are examining the effect of NF1 knockdown (KD) in human mammary epithelial cells (HMECs) derived from breast mammoplasty procedures. This study will provide information on the molecular mechanisms of breast cancer in NF1 deficient human subjects and will help to determine guidelines for the screening of NF1 breast cancer patients.

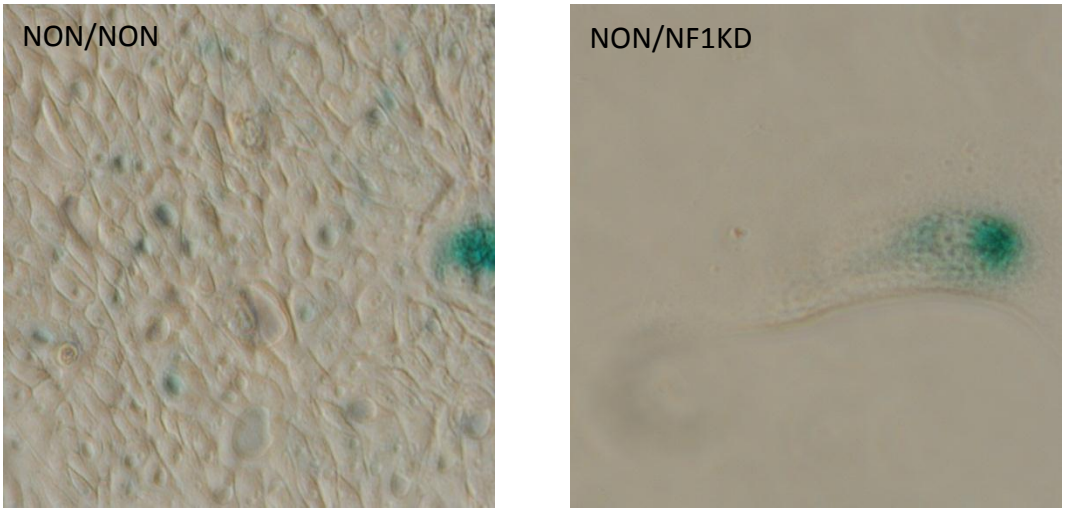
It has been shown that ablation of NF1 results in activation of Ras oncogene and that such activation results in transformation or senescence in different cell types. We performed experiments to determine whether these mechanisms could be induced in HMECs by an NF1 deficiency and the involvement of RB1 and p53 pathways.

NF1 knock out triggers cellular senescence in HME cells.

In order to study NF1 knock down in HMEC, we first had to establish conditions for the experiment using shRNAmir-NF1 (Openbiosystems, ThermoFisher Scientific). We created the lentiviral vector upon transfection of plasmids + shRNA into packaging cell line 293T, determined the titer of the virus and the infection efficiency. Each double infection was performed within 24 hours incubation from the first infection. The control cells were infected with the empty lentivirus (NON).

Same number of HME cells were plated before infection and a β-Gal assay was performed after 2 weeks of selection in puromycin. Fig. 1 shows the dramatic difference in cell number and phenotype between the control cells, NON/NON and the NF1 knockdown cells (NON/NF1 KD). Evidently the NF1 KD cells stop growing and undergo senescence. Senescent cells undergo phenotypic changes as they are enlarged and flattened and show increased B-Gal activity (blue cells).

Fig.1



Infections were repeated 3 times with slightly different conditions, but they always gave the same result: HMEC growth arrest and early senescence after depletion of NF1.

The NF1 knockdown was checked by western blot (Fig2) and the relative protein amounts were calculated in comparison with tubulin amounts (Graph 1). The decrease in NF1 expression is about 1/10 compared to the control.

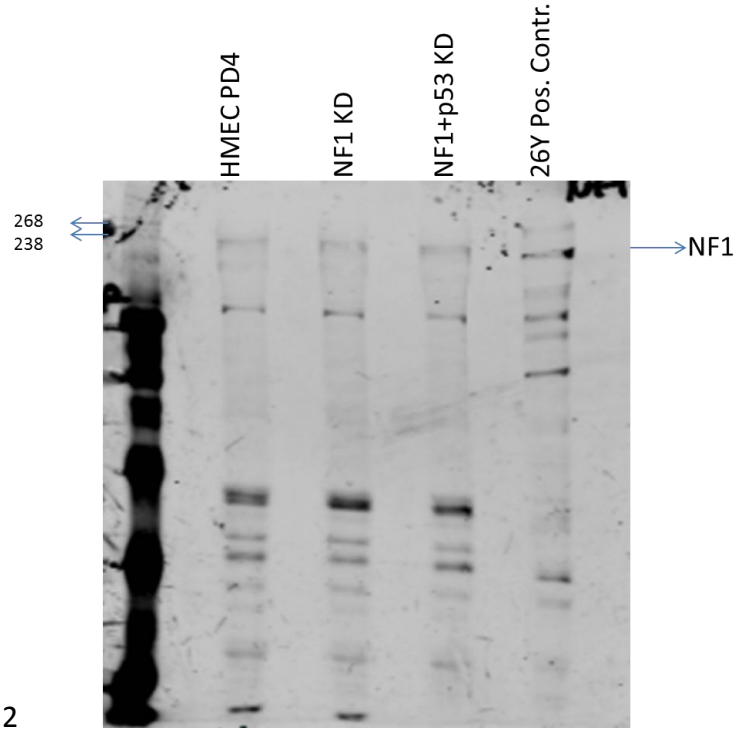
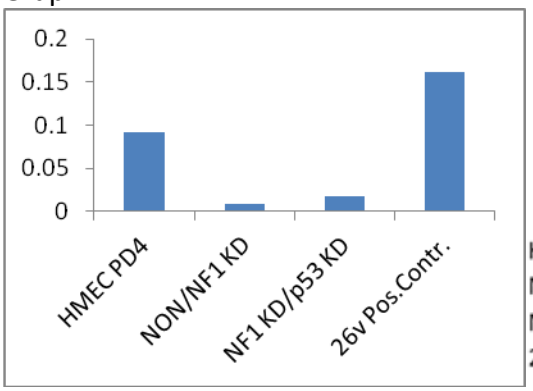


Fig.2

Graph 1



HMEC PD4	0.091048
NON/NF1 KD	0.008984
NF1 KD/p53 KD	0.017496
26v Pos.Contr.	0.16219

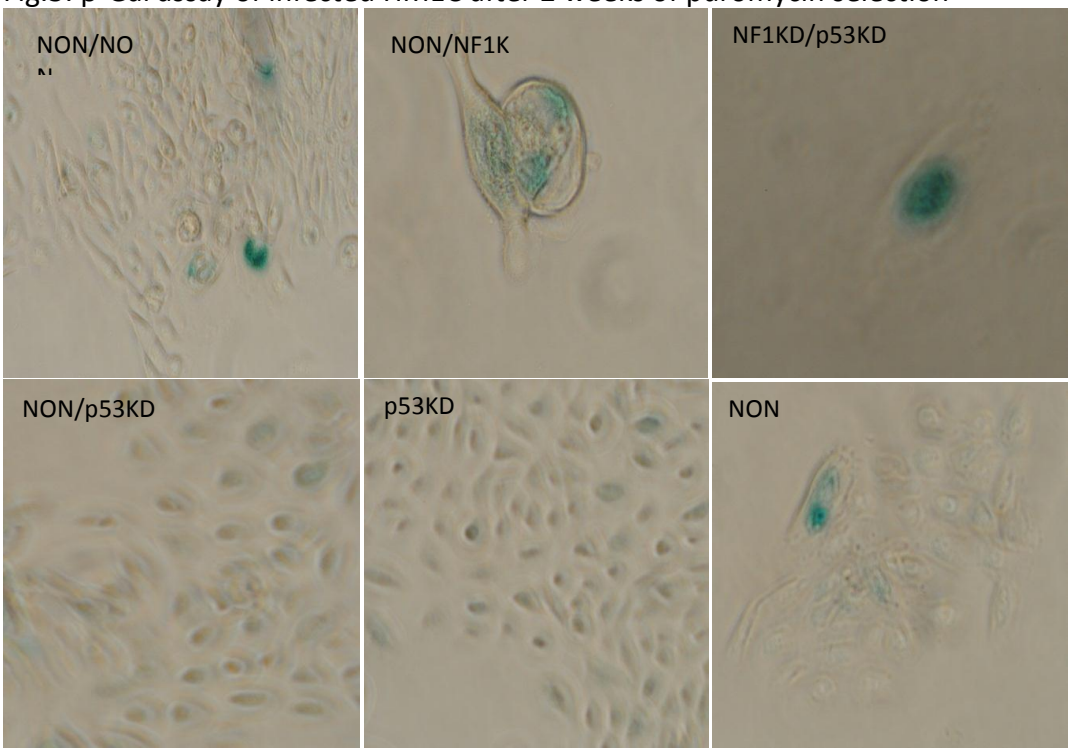
Loss of p53 or RB does not rescue senescent NF1 KD HMEC

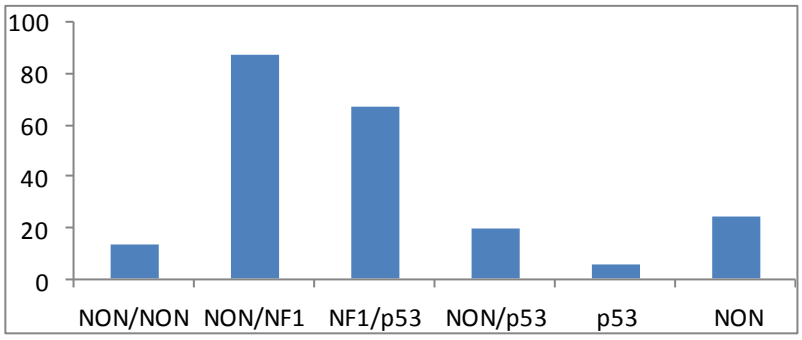
It is known that ablation of NF1 results in activation of Ras oncogene in mouse embryonic fibroblast (MEF) and such activation might trigger an oncogene induced senescence in cells (OIS). Known regulators of senescence are p53 and/or RB pathways, but other signals might be involved to suppress the oncogenic expression and induce senescence.

In order to understand the mechanisms that trigger senescence in HMEC after NF1 depletion and the involvement of p53 and/or RB in such process, we also prepared lentiviruses sh-RNA-p53 and sh-RNA-RB1.

The knock down of p53 alone in HMEC resulted in a temporary cell growth advantage for the first 2 weeks of culture when compared to the controls. However we observed an increased rate of cell death. After 4 weeks of cell culture, HMEC p53 KD became senescent and appeared not much different from the control cells (Fig.3), confirming data already published by Garbe et al, 2007. The double knockdown of p53 and NF1 in HMEC (NF1 KD/p53 KD) shows early senescence when compared to the HMEC control (NON/NON) and it is not able to rescue the cells from the senescent phenotype caused by NF1 knock down alone (Fig.3 and Graph2).

Fig.3: β-Gal assay of infected HMEC after 2 weeks of puromycin selection





Graph 2: Percentages of blue cells obtained after performing β-Gal Assay of infected cells

Interestingly the knockdown of RB1 in HMEC gave a growth advantage to NF1 KD HMEC for 14 days after performing the infections. Graph 3 shows the reduction in cell number after 7 days selection. HMEC NF1 KD infected with RB KD retained a higher number of cells compared to the NON/NF1 KD, suggesting a growth advantage and the prevention of senescence in the cells where RB is less expressed. Unfortunately when the cells were detached from the original plate and passed to new plates we observed a very high rate of cell death and the formation of few clones (Fig.4). However after a month in culture, the cells were not able to grow and the density and the phenotype of the cells were the same throughout different infections including the control (Fig.5). We grew the cells for an additional 3 weeks without signs of cell growth in any infections.

Graph 3

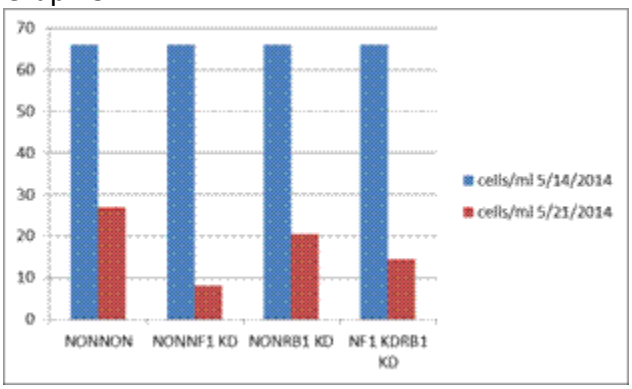
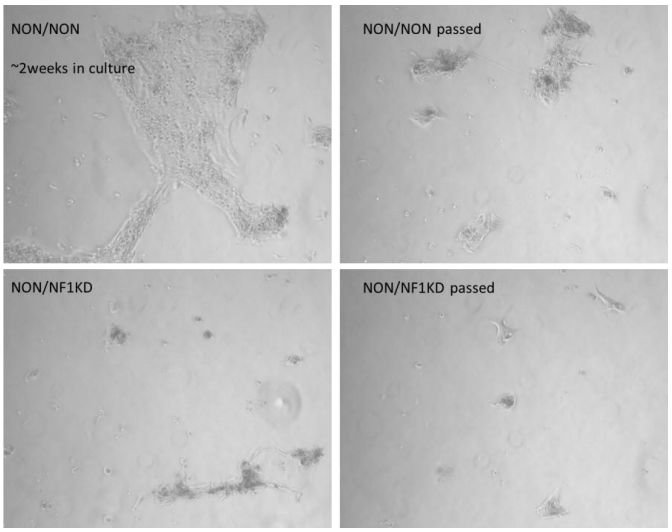


Fig. 4



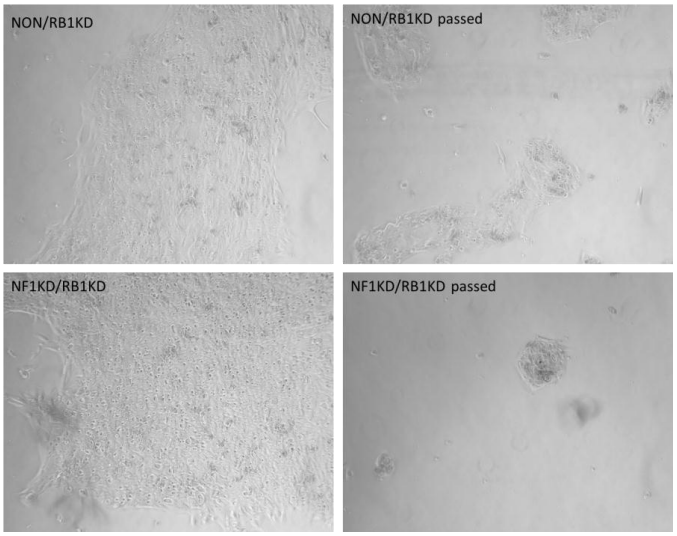
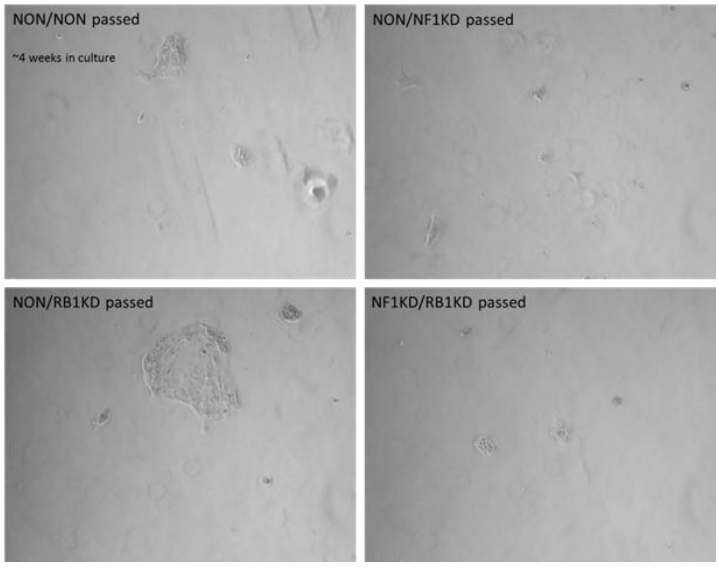


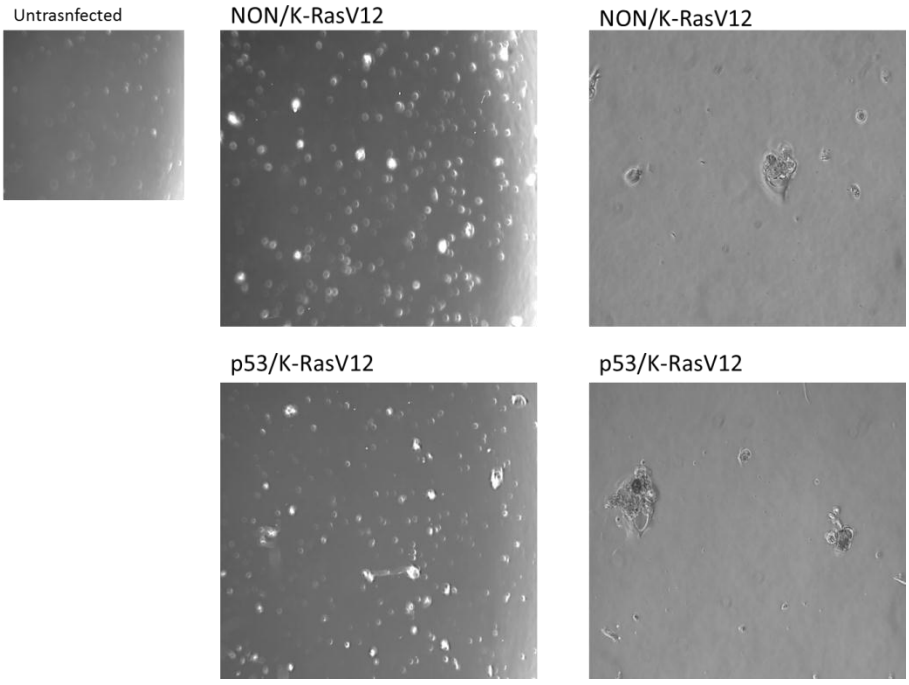
Fig. 5



Overexpression of mutant Ras does not result in immortalization /transformation of HMEC

It has been shown that MCF10A escape OIS when transfected with mutated Ras(V12) by degrading C/EBPbeta1 and the cells become transformed. To assess the sensitivity of HMEC cells to mutated Ras, Ras (V12) was overexpressed in HMEC NON and HMEC p53KD by plasmid transfection using the X-tremeGENE 9 DNA transfection reagent (Roche). Even in the cells where p53 expression was reduced, KRas (V12) overexpression did not give a growth advantage to the cells (Fig.6).

Fig.6



Conclusions

- NF1 inactivation results in HMEC senescence
- p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC.

  p53 inactivation provides an initial growth advantage to HMEC with a consequent large number of cell death confirming data already published (Garbe et al 2007)
- RBKD HMEC show higher growth rate and a decreased cell death compared to p53KD HMEC
- Overexpression of K-Ras V12 does not transform p53 inactivated HMEC

Plans

NF1KD, P53KD and RBKD will need to be confirmed by Western and/or RT-PCR. Low levels of p53 and RB inactivation might be an explanation to the fact that we are not able to block the senescent phenotype of HMEC NF1KD.

Ras and Ras effector activity will need to be confirmed in HMEC cells and HMEC infected cells as well.

Further studies are needed to understand which of Ras negative regulators might be also involved in the OIS of HMEC NF1KD. It might be worth to study the process of HMEC induced senescence in the immortalized cell line, such as hTERT-HMEC.

KEY RESEARCH ACCOMPLISHMENTS

The strong association between family history of cancer and occurrence of breast cancer in women with NF1 highlights the importance of gathering comprehensive family history, especially family cancer history, during the clinical encounters. Family history of cancers should be used as a tool to assess breast cancer risk in women with NF1.

Women with NF1 have inadequate breast cancer screening or evaluation. Patients and care providers should be educated about moderately increased breast cancer risk in NF1. MR imaging for breast lumps should be used more frequently to decrease the need for biopsy in order to differentiate cancer versus neurofibroma in the breasts.

CONCLUSIONS

1. Twenty out of 423 (4.73%) NF1 women have a diagnosis of breast cancer. This is higher than expected (two institutions, Henry Ford Health System and Johns Hopkins University have published these data individually in 2012). The pathological types of breast cancers are not much different than the general population.

2. Family history of cancer is strongly associated with breast cancer occurrence in NF1 women ($p=6.05x10^{-5}$). However family history of NF1 is not associated with personal history of breast cancer. This indicates that breast cancer in women with NF1 may be a result of additional familial germline risk factor. This risk factor may require NF1 gene mutation to manifest its effect. The upcoming analysis of whole exome sequencing data may identify additional germline risk factor. Never the less, an alternative explanation could be that this familial risk factor is environmental exposure which is familial or geographically specific. Family history of cancers should be used as a tool to assess breast cancer risk in women with NF1. The future goal is to quantify the elevation of the risk based on family history so that properly targeted breast cancer screening can be recommended.

3. Women with NF1 have inadequate breast cancer screening or evaluation. Patients and care providers should be educated about moderately increased breast cancer risk in NF1. MR imaging for breast lumps should be used more frequently to decrease the need for biopsy in order to differentiate cancer versus neurofibroma in the breasts.

4. More NF1 women with plexiform neurofibromas (PN) developed MPNST than those without PN; 7.91 % v.s. 3.14%; p=0.049. This finding consists with previous knowledge that most of the MPNST have developed from existing PNs (Tucker T et al., 2005).

5. More women with learning disability have a history of (CNS) brain tumor and/or optic glioma (OPG) than those without tumor, 23.3% v.s. 11.23%; p=0.012. Optic glioma mostly occurs in the early childhood. Treatment for OPG, such as chemotherapy or radiation therapy is known to have adverse effects on the developing brain. In this study, the history of OPG treatment was not collected. We cannot determine the level of which OPG treatment contributed to the learning disability based on this study. Future study may be targeted at clarifying this question. If learning disability is proven to be an indicator for increased risk for brain tumor, it can be used to stratify the risk and help to develop screening recommendation for CNS tumor. Discovering potential molecular basis for this association may even aid in CNS tumor therapy in the future.

6. European Americans (EA) are more likely to develop CNS tumor and/or OPG than African Americans (AA); 21.24% v.s. 6.82%; p=0.002. The rate of OPG alone is

also higher in EA than AA; p=0.013. This supports the previous report that there is a disproportionally high rate of CNS tumor or OPG in EA population (Robertson JT et al., 2002; Saal HM et al., 1995; Pletcher BA et al., 1996).

7. Breast cancer is seen slightly more in AAs than EAs, 6.78% v.s. 4%, without statistical significance. However, more EAs were affected with other cancers than AAs; 25.2% v.s. 15.25%; p=0.032.

8. Out of 14 NF1 women affected with breast cancer, 4(28.6%) have in-frame splicing mutation, which is more than the 6.5% reported in a previous large French cohort NF1 study (Sabbagh et al., 2013).

9. NF1 inactivation results in human mammary epithelial cells (HMECs) senescence; p53 inactivation does not rescue the senescence phenotype in NF1KD (knockdown) HMEC; p53 inactivation provides an initial growth advantage to HMEC with a consequent large number of cell death; Overexpression of K-Ras V12 does not transform p53 inactivated HMEC. Further studies are needed to understand which of Ras negative regulators might be also involved in the OIS of HMEC NF1KD. It might be worth to study the process of HMEC induced senescence in other immortalized cell line.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

None

INVENTIONS, PATENTS, AND LICENSES

None

REPORTABLE OUTCOMES

OTHER ACHIEVEMENTS

None

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APPENDICES

None